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Biosynthesis of the Vitamin E Compound δ-Tocotrienol in Recombinant *Escherichia coli* Cells

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The biosynthesis of natural products in a fast growing and easy to manipulate heterologous host system, such as Escherichia coli*, is of increasing interest in biotechnology. This procedure allows the investigation of complex natural product biosynthesis and facilitates the engineering of pathways. Here we describe the cloning and the heterologous expression of tocochromanol (vitamin E) biosynthesis genes in* E. coli*. Tocochromanols are synthesized solely in photosynthetic organisms (cyanobacteria, algae, and higher green plants). For recombinant tocochromanol biosynthesis, the genes encoding hydroxyphenylpyruvate dioxygenase (*hpd*), geranylgeranylpyrophosphate synthase (*crtE*), geranylgeranylpyrophosphate reductase (*ggh*), homogentisate phytyltransferase (*hpt*), and tocopherol-cyclase (*cyc*) were cloned in a stepwise fashion and expressed in* E. coli*. Recombinant* E. coli *cells were cultivated and analyzed for tocochromanol compounds and their biosynthesis precursors. The expression of only* hpd *from* Pseudomonas putida *or* crtE *from* Pantoea ananatis *resulted in the accumulation of 336 mgL^{-1} homogentisate and 84 μgL^{-1} geranylgeranylpyrophosphate in* E. coli *cultures. Simultaneous expression of* hpd, crtE*, and* hpt *from* Synechocystis *sp. under the control of single tac-promoter resulted in the production of methyl-6-geranylgeranyl-benzoquinol (67.9 μgg^{-1}). Additional expression of the tocopherol cyclase gene* vte1 *from* Arabidopsis thaliana *resulted in the novel formation of a vitamin E compound—δ-tocotrienol (15 μgg^{-1})—in* E. coli.

Introduction

Vitamin E is a generic term that describes a group of eight lipophilic compounds: the tocochromanols (four tocopherols and four tocotrienols).[1] Tocochromanols each consist of a polar aromatic head group derived from homogentisate (chromanol moiety), linked to a lipophilic isoprenoid-derived hydrocarbon tail. Depending on the state of saturation in the isoprenoid side chain and the methylation state of the aromatic ring, they are referred to as α–δ-tocopherol and α–δ-tocotrienol, respectively.[1] The tocotrienol side chain includes three *trans* double bonds. Tocochromanols are exclusively produced by photosynthetic organisms (eukaryotic algae and green plants, some prokaryotic cyanobacteria, such as *Synechocystis*)[2] and are potent antioxidants that protect the photosynthetic organism against reactive oxygen species.[3] The biopotency of tocochromanols for animals and humans is expressed as vitamin E activity; (*R*,*R*,*R*)-α-tocopherol was found to have the highest vitamin E activity in vivo.[2b–c,4] Besides their vitamin E activity, tocochromanols are also associated with numerous additional conditions in humans, including immune response, cellular signaling, reproduction, anticancer activity, and cardiovascular benefits.[1,4,5] Tocotrienols have been receiving increased attention since reports of biological activities distinct from those of α-tocopherol in health and disease (neuroprotective, antioxidant, anticancer, and cholesterol-lowering properties).[6]

Chemical synthesis of vitamin E mainly yields all-*rac* α-tocopherol.[7] Although the asymmetric synthesis of the natural tocopherol and tocotrienol isomers has been achieved,[8] currently the enantiomerically pure vitamin E compounds—for use as, for example, food additives—are extracted and isolated from natural sources.

The biosynthesis pathway of tocochromanols in green plants and cyanobacteria has largely been determined by analysis of mutant organisms and by in vitro characterization of the biosynthesis enzymes.[9] The precursor for the chromanol head is homogentisate, which is derived from tyrosine (or generally from the shikimate pathway) through *p*-hydroxyphenylpyruvate (HPP), which is oxidized to homogentisate (HGA) by *p*-hydroxyphenylpyruvate dioxygenase (HPD).[10] The other vitamin E precursor, geranylgeranylpyrophosphate (GGPP), is synthesized by the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway[9,11] (Scheme 1). The final step in the formation of GGPP requires the condensation of farnesylpyrophosphate (FPP) and isoprenylpyrophosphate (IPP) by geranylgeranylpyrophosphate synthase (CrtE).[12] GGPP is either used directly for synthesis of

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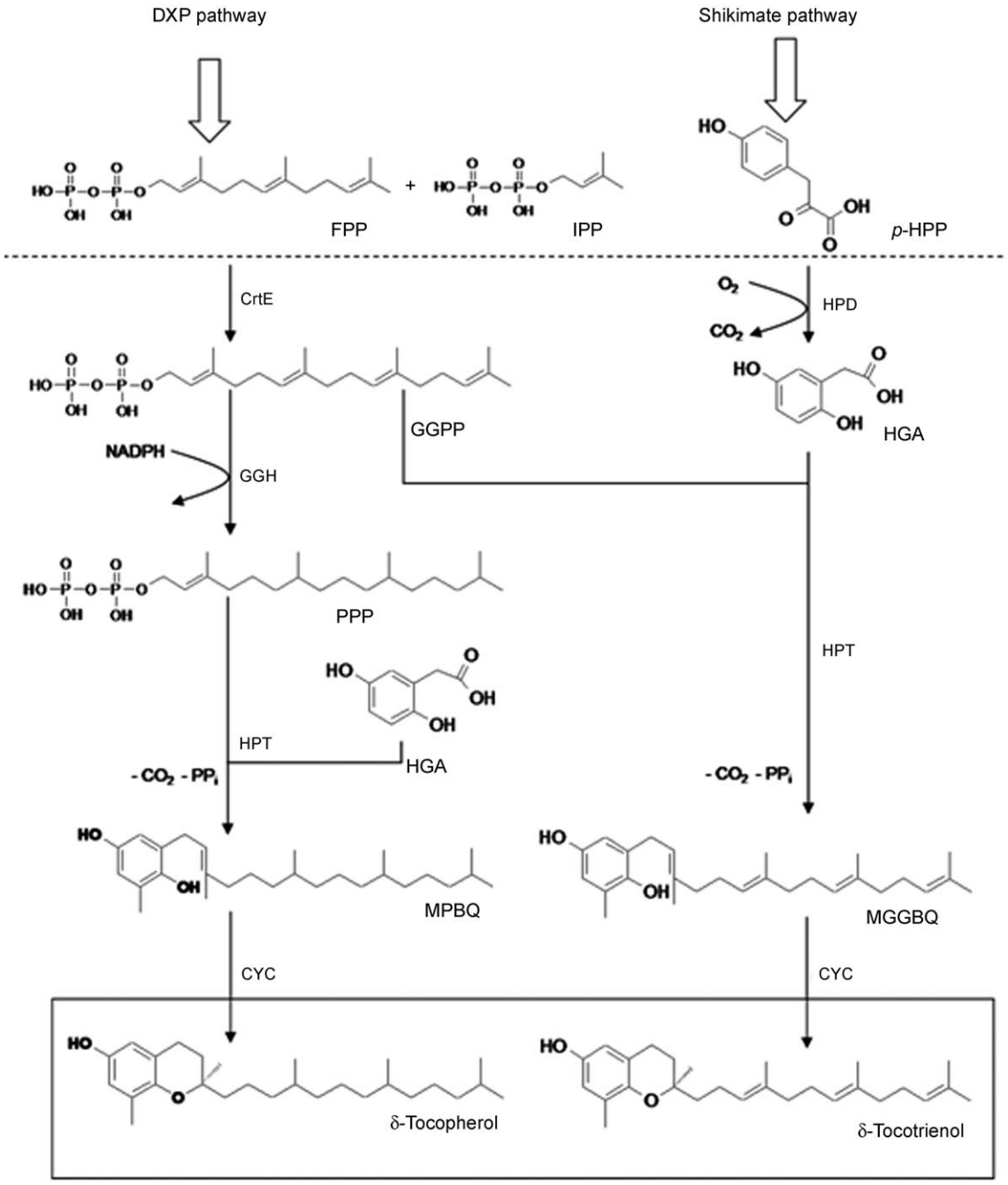

Scheme 1. Proposed pathway for δ-tocochromanol biosynthesis in recombinant *Escherichia coli*. Steps of the existing DXP and shikimate pathways are shown until the horizontal dotted line. Enzymes catalyzing the individual novel steps are denoted by bold letters. Pathway intermediates are denoted as FPP (farnesylpyrophosphate), *p*-HPP (*p*-hydroxyphenyl-pyruvate), GGPP (geranylgeranylpyrophosphate), PPP (phytylpyrophosphate), HGA (homogentisic acid), MPBQ (2-methyl-6-phytyl-benzoquinol), and MGGBQ (2-methyl-6-geranylgeranyl-benzoquinol). GGPP and PPP combine with HGA through condensation and decarboxylation to produce MGGBQ and MPBQ, respectively. Cyclization of these intermediates then yields δ-tocotrienol and δ-tocopherol, respectively.

tocotrienols or is further reduced by a geranylgeranylpyrophosphate reductase (GGH)[13] to phytylpyrophosphate (PPP). In plants, the transfer of PPP or GGPP to HGA is catalyzed by a homogentisate-phytyltransferase (HPT) or by a homogentisate-geranylgeranyltransferase (HGGT, in monocots).[14] In contrast, the cyanobacterium *Synechocystis* contains only one transferase enzyme (HPT, alias SLR1736), which is able to combine PPP and GGPP with HGA, PPP being the preferred substrate.[13c, 14c] The resulting benzoquinol compounds—2-methyl-6-geranyl-geranyl-benzoquinol (MGGBQ) and 2-methyl-6-phytyl-benzoquinol (MPBQ), respectively—are then methylated by a prenyl-benzoquinol methyltransferase to yield the tocochromanols.[9] Cyclization of the dimethyl compound by tocopherol/tocotrienol-cyclase leads to the formation of γ-tocochromanol, and the cyclization of MGGBQ and MPBQ to δ-tocochromanol, respectively.[10] Methylation of γ- or δ-tocochromanol by the tocopherol/tocotrienol-methyltransferase finally results in the formation of α- or β-tocochromanol, respectively.[9]

There are many examples of the heterologous synthesis of rare natural products, such as polyketides, isoprenoids, alkaloids, and flavonoids, in microorganisms, of which *E. coli* has been shown to be a good host organism because it is genetically well characterized, fast growing, and is easy to manipulate genetically.[15] So far, biosynthesis of tocochromanols in a nonphotosynthetic organism has not been reported. Here we present the heterologous expression of tocochromanol biosynthesis genes in the non-photosynthetic bacterium *E. coli* K-12, which lacks the necessary gene functions past the precursor molecules FPP and HPP.[16] We have cloned the biosynthesis genes for the production of δ-tocotrienol in *E. coli* and show that the joint expression of the genes *hpd*, *crtE*, *hpt*, and *cyc* leads to the heterologous biosynthesis of a vitamin E compound in a non-photosynthetic host organism (Scheme 1). This opens up the opportunity for further metabolic engineering of tocochromanol pathways in *E. coli* cells.

Results

In vivo synthesis of the vitamin E precursors HGA and GGPP

In order to produce the precursor homogentisate in *E. coli* we cloned the corresponding gene (*hpd*) for a *p*-hydroxyphenylpyruvate-dioxygenase from *Pseudomonas putida* KT2440 into the expression vector pJF119ΔN (pCAS2JF, Table 1) under the control of the IPTG-inducible *tac* promoter. After induction, *E. coli* strain DH5α/pCAS2JF formed HGA as major compound in supernatants or in the cell pellets (Figure 1). HGA was found in both growth media: LB or minimal medium produced 2 mM or 0.2 mM of HGA, respectively. Addition of tyrosine to the minimal medium led to an increase in HGA formation in the supernatant to about 2 mM. After prolonged incubation (>40 h) the concentration of free HGA had decreased due to the formation of brown ochronotic pigment, which is due to the spontaneous oxidation and polymerization of HGA.[17] No HGA was found in the control strain *E. coli* DH5α/pJF119ΔN.

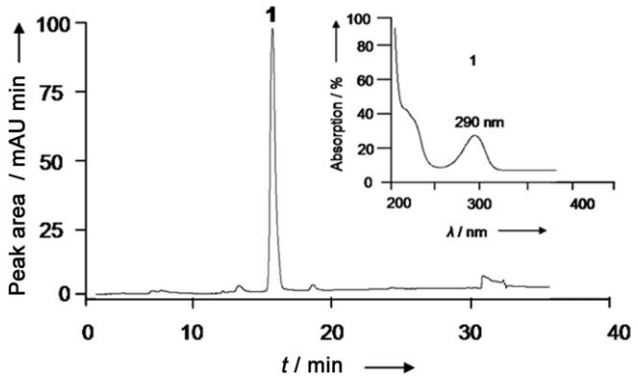

Figure 1. HPLC chromatogram illustrating HGA formation in recombinant *E. coli* shown on the left side. HGA released into the supernatant of the growth medium was injected for HPLC analysis. Peak 1 shows HGA produced during cultivation of strain *E. coli* DH5α carrying plasmid pCAS2JF. The product peak 1 has the same retention time and the same maximum UV absorption (290 nm) as the standard HGA. The maximum UV absorption is shown as inset.

Table 1. Strains and plasmids used in this work.

Strain or plasmid	Relevant properties or genotype	Source
Strains		
E. coli DH5α	F⁻, ϕ80d, *lacZ*ΔM15, *endA*1, *recA*1, *hsdR*17-($r_K^-m_K^-$), *supE*44, *thi*-1, *gyrA*96, *relA*1, Δ(*lacZYA-argF*)U169	[a]
E. coli M15 [pREP4]	F⁻, *lacZΔM15, thi*-1, *lac*⁻, *mtl*⁻, *recA*⁺, Km^R	[a]
Pseudomonas putida KT2440	type strain	[b]
Synechocystis sp. PCC 6803	type strain	[c]
Plasmids		
pCAR16	β-carotene biosynthesis gene cluster from *Pantoea ananatis* in pUC19, Amp^R	[27]
pJF119EH	cloning vector, tac-promoter, IPTG inducible, Amp^R	[25]
pJF119ΔN	cloning vector, RBS, tac-promoter, IPTG inducible, Amp^R	this study
pAW229	cloning vector, RBS, *rha*-promoter, L-rhamnose inducible, Cm^R	[26]
pQE31-VTE1	tocopherol-cyclase (vte1) from *Arabidopsis thaliana* in pQE31, IPTG inducible, Amp^R	[18a]
pCAS2JF	1.2 kb EcoRI/BamHI PCR fragment *hpd* in pJF119EH	this study
pCAS8	1.1 kb NdeI/BamHI PCR fragment *ggh* in pJF119ΔN	this study
pCAS11	0.9 kb BglII/BamHI PCR fragment *crtE* in pCAS8	this study
pCAS12	1.0 kb BglII/BamHI PCR fragment *hpt* in pCAS11	this study
pCAS15	1.2 kb NdeI/BamHI PCR fragment *hpd* in pAW229	this study
pCAS18	1.2 kb BglII/BamHI PCR fragment *hpd* in pCAS11	this study
pCAS19	1.0 kb NdeI/BamHI PCR fragment *hpt* in pAW229	this study
pCAS23	1.0 kb NdeI/BamHI PCR fragment *hpt* in pJF119ΔN	this study
pCAS24	0.9 kb NdeI/BamHI PCR fragment *crtE* in pCAS23	this study
pCAS27	1.1 kb NdeI/BamHI PCR fragment *cyc-sy* in pAW229	this study
pCAS29	1.2 kb BglII/BamHI PCR fragment *hpd* in pCAS18	this study
pCAS30	0.9 kb NdeI/BamHI PCR fragment *crtE* in pJF119ΔN	this study
pCAS37	1.2 kb BglII/BamHI PCR fragment *hpd* in pCAS24	this study
pCAS47	1.3 kb BglII PCR fragment *vte1* in pCAS29	this study

[a] Commercial strain. [b] Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). [c] A kind gift from Prof. Forchhammer, Universität-Gießen (Germany).

For the production of the isoprenoid precursor GGPP, the gene *crtE* encoding geranylgeranylpyrophosphate synthase from *Pantoea ananatis* was subcloned (Table 1). *E. coli* DH5α/pCAS30 (pJF119ΔN+*crtE*) was analyzed for the formation of FPP and GGPP after cultivation and induction in LB medium containing 2% glycerol. Cells were extracted, pyrophosphate compounds were dephosphorylated with alkaline phosphatase, and the corresponding alcohols were extracted in situ by use

of a two-phase system. The dephosphorylation products—farnesol and geranylgeraniol, respectively—were identified and quantified by GC-MS. Cells of *E. coli* DH5α/pCAS30 had accumulated 188 ± 14 nM (84.4 μg L^{-1}) GGPP after 52 h of cultivation (Figure 2). FPP was not detected in these cells. No GGPP was detected in control strains with the empty vector, pJF119ΔN, whereas FPP was found in these cells (up to 32 ± 4 nM or 12.2 μg L^{-1}).

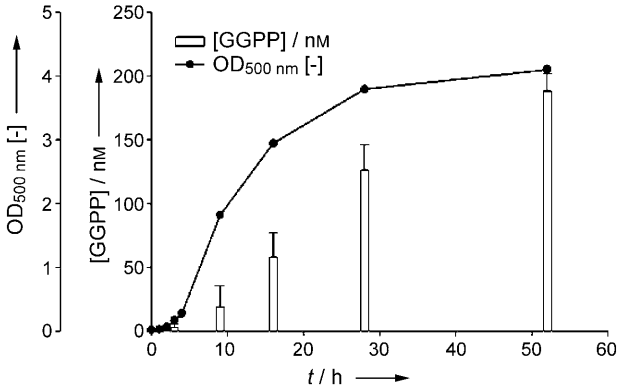

Figure 2. Growth curve of—and GGPP production by—*E. coli* DH5α strain carrying plasmid pCAS30 in LB medium supplemented with glycerol (2%, *v*/*v*) and ampicillin (100 mg L^{-1}). The black filled circles represent the optical density at 500 nm. GGPP extracted from cells is represented by bars.

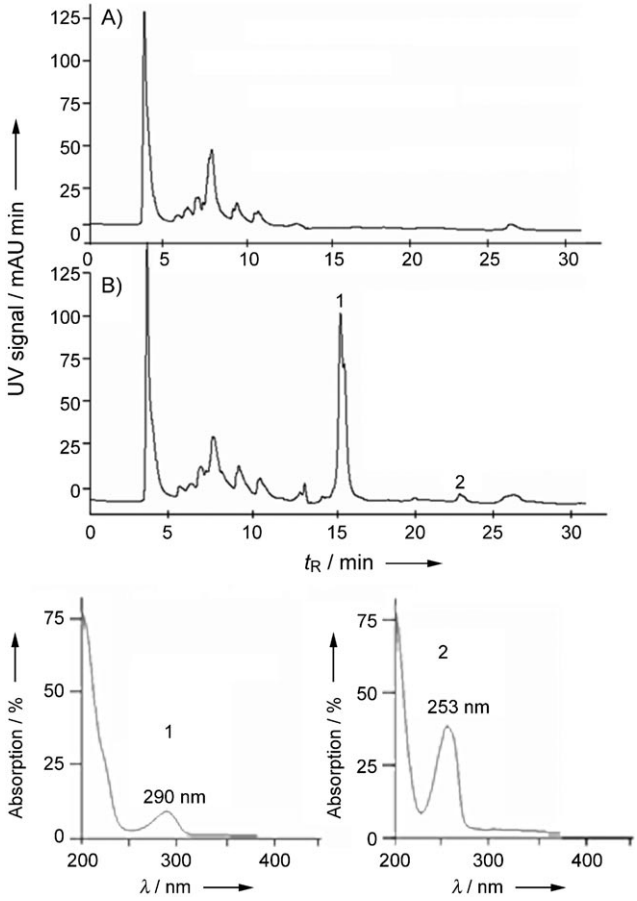

Figure 3. HPLC analysis of MGGBQ-containing extracts from recombinant *E. coli*. The chromatograms shown in this figure are only qualitative representations of the samples. The *y*-axis represents the UV signal in mAU min, the *x*-axis represents the retention time in min. A) HPLC chromatogram for extract from *E. coli* DH5α carrying plasmid pCAS18. No MGGBQ either in reduced or oxidized form was observed. B) HPLC chromatogram for extract from *E. coli* DH5α carrying plasmid pCAS29. Peak 1 represents MGGBQ in its reduced form and peak 2 represents MGGBQ in its oxidized form, shown on left hand side. On the right hand side the maximum UV absorbance for each individual peak is shown, with the *y*-axis being the absorption in % and the *x*-axis being the wavelength in nm. The reduced form of MGGBQ has a maximum absorbance at 290 nm and the oxidized form at 253 nm.

Gene cassette assembly and in vivo production of MGGBQ

Having shown that both HGA and GGPP were formed in recombinant *E. coli* cells, we combined the genes *hpd* and *crtE* with the genes *hpt* and *chlP* (=*ggh*) from *Synechocystis* sp. PCC6803. The genes were cloned into the expression vector pJF119ΔN in a stepwise manner so that each gene has its own Shine–Dalgarno sequence (GAAGGA) eight nucleotides upstream of the corresponding start codon (Table 1 and Supporting Information). The resulting plasmid pCAS29 was transformed and expressed in *E. coli* DH5α. Cell-free culture broth and cell pellets of cultures (LB-glycerol medium, induction times from 4 to 72 h) were extracted into organic solvents and analyzed by HPLC. As control, *E. coli* DH5α transformed with vector pCAS18 (encoding *chlP*, *crtE*, and *hpd,* but lacking *hpt*) was cultivated under the same conditions. Comparison of both cell extracts by HPLC analysis revealed an additional peak, with an absorption maximum at 290 nm, in the extracts of *E. coli* DH5α/pCAS29 (Figure 3). LC-MS analysis revealed a product mass of 397 $[M+H]^+$, 414 $[M+NH_4]^+$, which corresponds to the mass of MGGBQ (see the Supporting Information). After oxidation with silver oxide (Ag_2O), the extract containing the benzoquinol compound MGGBQ displayed complete conversion into its benzoquinone form, showing a characteristic shift of the absorption maximum to 253 nm. MS analysis of the oxidized MGGBQ gave the expected masses of 395 $[M+H]^+$, 412 $[M+NH_4]^+$ (Supporting Information). The proportion of the oxidized MGGBQ form in the extracts of *E. coli* DH5α/pCAS29 was low, less than 5%. MPBQ, however, which we had expected to find, was not detectable in the extracts of *E. coli* DH5α/pCAS29, so the activity of the *chlP* (=*ggh)* gene product geranylgeranylpyrophosphate reductase was in doubt. However, we confirmed that all four recombinant genes in pCAS29 had been transcribed (transcript analysis by RT real-time PCR) and that recombinant proteins of the expected size were formed (SDS-PAGE and 2D gel electrophoresis; data not shown).

In further cultivations of *E. coli* containing *hpd*, *crtE*, and *hpt* (pCAS15+pCAS24, or pCAS37) the same product MGGBQ was extracted from the cell pellet. Again, the isolation of phytylpyrophosphate (PPP) after cultivation and induction of *E. coli* DH5α/pCAS11 (expressing *crtE* and *ggh* alias *chlP*) failed, only GGPP was detected in these cultures (data not shown). We concluded that the in vivo enzyme activity of GGH/ChlP in *E. coli* might be insufficient for production of PPP.

Cloning of a tocopherol/tocotrienol-cyclase and in vivo production of δ-tocotrienol

The biosynthesis of tocochromanols requires the intramolecular cyclization of the corresponding benzoquinol. We therefore wanted to complete the biosynthesis pathway in *E. coli* through the addition of a cyclase gene to the artificial gene cassette of plasmid pCAS29. As the *cyc* gene of *Synechocystis* did not lead to the formation of δ-tocotrienol in vivo (only MGGBQ was found in the pellet fraction; data not shown), we turned to the cloning of the recently described *vte1* gene from *Arabidopsis thaliana*.[18] The VTE1 enzyme had been shown by in vitro assays to convert MPBQ and DMGGBQ into the corresponding tocochromanol compounds.[18a] The protein shows 41% amino acid identity to the Cyc protein from *Synechocystis*.[18a] Because activity with the substrate MGGBQ had not yet been shown, we first tested the activity of the recombinant enzyme in vitro.

With MGGBQ, isolated from *E. coli* DH5a/pCAS29 cultures, and purified His-tagged VTE1, up to 90% of the substrate was converted into δ-tocotrienol in vitro within 35 h (Figure 4). The product was identified by LC-MS and by HPLC co-injection of the δ-tocotrienol standard. In the control experiments, without addition of recombinant VTE1, no conversion of MGGBQ was detectable by HPLC analysis.

We integrated the *vte1* gene into the gene cassette on plasmid pCAS29 (Table 1). *E. coli* cells containing plasmid pCAS47 yielded δ-tocotrienol after induction by IPTG (HPLC analysis in Figure 5 and LC-MS in the Supporting Information). The maximum of MGGBQ concentration ($332.1 \pm 14.2\ \mu g\,g^{-1}$ cellular dry weight (CDW)) was achieved 18 h after the start of the induction, with small quantities of δ-tocotrienol ($2.6 \pm 0.2\ \mu g\,g^{-1}$ CDW) being detectable. Over time, the MGGBQ concentration slowly decreased, with a concomitant increase in δ-tocotrienol concentration. Cultures had reached the stationary phase (OD_{600} of 2.4) 30 h after induction. Maximal δ-tocotrienol production was seen during the stationary phase (up to $15.0 \pm 1.1\ \mu g\,g^{-1}$ CDW after 48 h of induction), with MGGBQ concentration levels dropping to $87.3 \pm 1.8\ \mu g\,g^{-1}$ CDW (Figure 6). The control strain *E. coli* DH5α/pCAS29 was cultivated in parallel and showed a maximum concentration of MGGBQ ($67.9 \pm 2.2\ \mu g\,g^{-1}$ CDW) after 24 h of induction with a subsequent

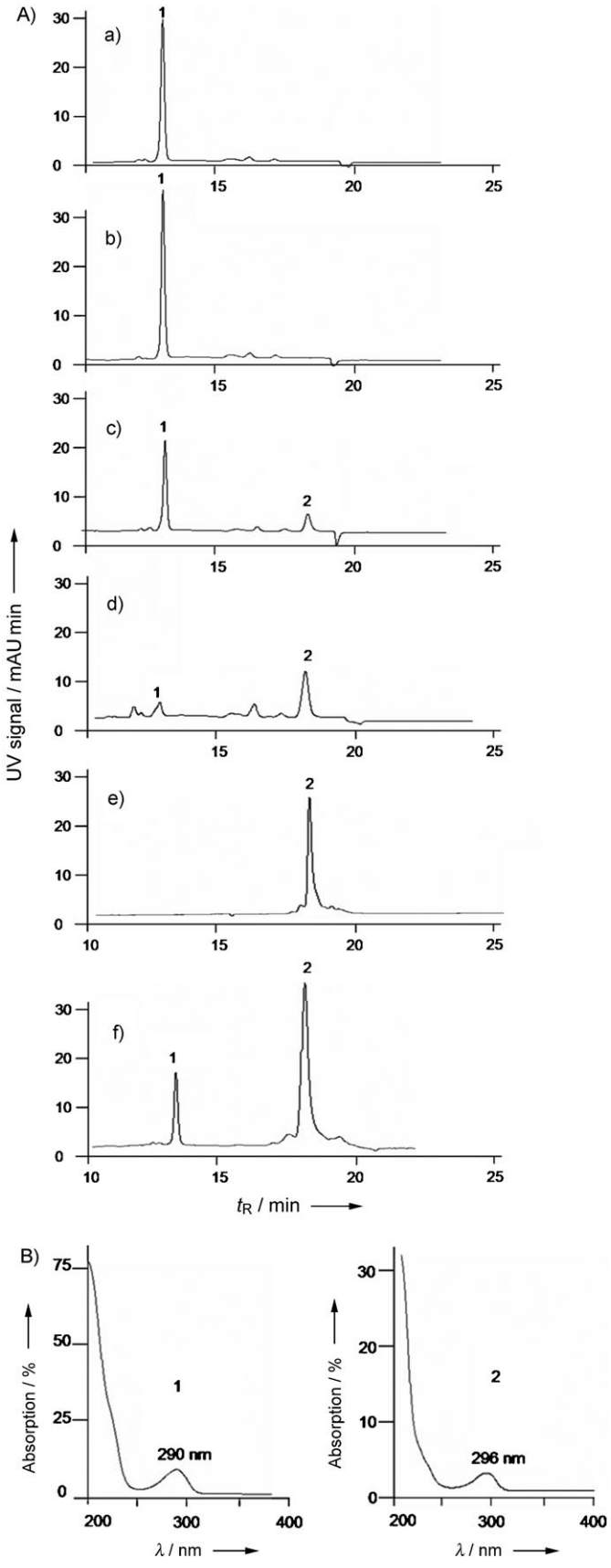

Figure 4. In vitro δ-tocotrienol assay. A) HPLC chromatograms of different samples from in vitro δ-tocotrienol assay. The chromatograms shown in this figure are only qualitative representations of the samples. Purified MGGBQ was the substrate used in this activity assay (a–d). a) Control reaction carried out without proteins. Unreacted MGGBQ represented by peak 1, with no new peak after 35 h of incubation. b) Reaction carried out with purified His-Tag TC proteins (10 μg; see the Experimental Section for details). MGGBQ represented by peak 1 after 0 h of incubation. c) Reaction products after 11 h of incubation showing unreacted MGGBQ and a new peak (δ-tocotrienol; peak 2). d) Reaction products after 35 h of incubation: 90% of the MGGBQ had been consumed, and 0.405 μg of δ-tocotrienol, represented by peak 2, had been produced. e) δ-Tocotrienol HPLC standard. f) Reaction product after 11 h of incubation (i.e., c), co-injected with δ-tocotrienol standard. B) Graph showing maximum UV absorption for each peak. Peak 1 represents the MGGBQ in reduced form, which has a maximum UV absorption at 290 nm. Peak 2 represents the δ-tocotrienol, which has a maximum UV absorption at 296 nm

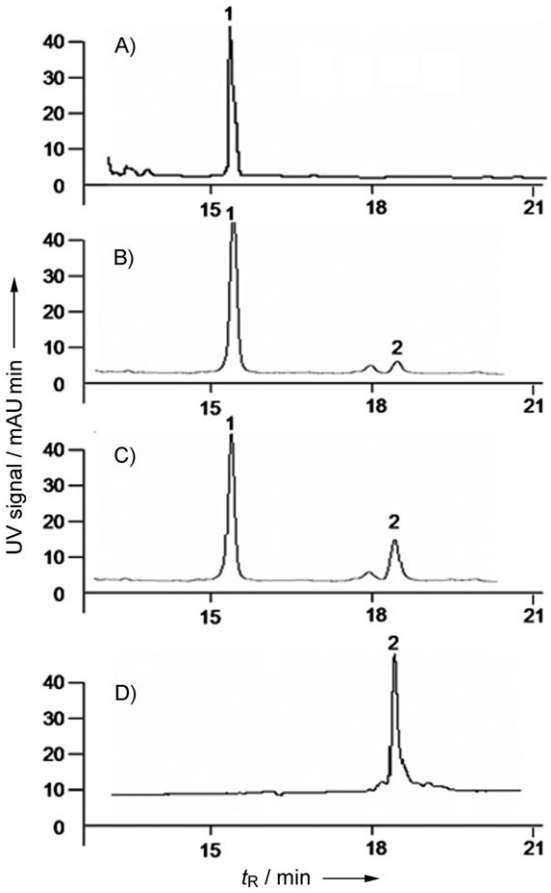

Figure 5. δ-Tocotrienol from recombinant *E. coli* cells (HPLC analysis). The chromatograms shown in this figure are only qualitative representations of the samples. The *y*-axis represents the UV signal in mAU min, the *x*-axis represents the retention time in minutes. A) HPLC chromatogram for extracts from *E. coli* DH5a carrying plasmid pCAS29. Peak 1 represents MGGBQ in its reduced form. No signal for δ-tocotrienol was detected. B) HPLC chromatogram for extracts from *E. coli* DH5a carrying plasmid pCAS47. Peak 1 represents MGGBQ (reduced form) and peak 2 represents δ-tocotrienol. C) HPLC chromatogram for extracts from *E. coli* DH5α carrying plasmid pCAS47—that is, (B)—coinjected with standard δ-tocotrienol. D) HPLC chromatogram of standard δ-tocotrienol represented by peak 2. Peak 2 has the same retention time as that of peak 2 shown in (B) The maximum UV absorption for peak 1 (MGGBQ in reduced form) is 290 nm and that of peak 2 (δ-tocotrienol) is 296 nm, the same as shown in Figure 4 B.2. The oxidized form of MGGBQ, which has a maximum absorption at 253 nm, was also detected in (a) and (b). This cannot be seen in chromatograms (a) and (b) because these chromatograms are measured at UV wavelength of 290 nm.

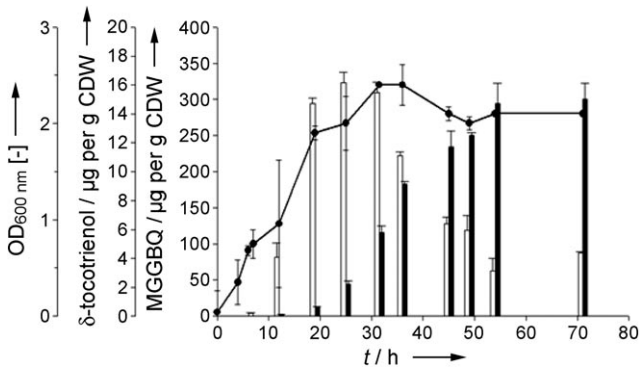

Figure 6. Growth curve of *E. coli* cells and their MGGBQ and δ-tocotrienol production. Growth curve represented by optical density measured at 600 nm (OD_{600}, ●) for δ-tocotrienol-producing strain *E. coli* DH5α carrying plasmid pCAS47 during a shaking flask experiment in LB medium supplemented with glycerol (2%, *v/v*) and ampicillin (100 µg mL^{-1}). Concentrations of extracted MGGBQ and δ-tocotrienol from cells, produced during cultivation. These are shown in the form of bar charts, empty bar charts representing MGGBQ (reduced form) concentrations and the black filled bar charts representing δ-tocotrienol concentrations. *E. coli* cells carrying plasmid pCAS29 were cultivated as a control strain in the same medium and under similar cultivation conditions. Only MGGBQ was detected in the cell extracts of the samples analyzed by HPLC. No δ-tocotrienol was detected in these samples. Data not shown.

gradual decrease thereafter (data not shown). We noted that this culture reached a higher optical density ($OD_{600\ nm}$ of 5.5, 60 h after induction) than the culture with the *cyc* (*vte1*) gene. This points to possible growth inhibition of strain DH5α/pCAS 47.

Discussion

The biosynthesis of tocochromanols in plants and in cyanobacteria has been revealed in previous works.[9b]

In our approach we cloned and expressed the genes for tocochromanol biosynthesis in *E. coli* in a stepwise fashion and analyzed the novel heterologous products. Because the vitamin E biosynthesis genes in the native vitamin E-producing organisms are not organized in one operon, we constructed an artificial gene cluster containing all genes believed to be involved in the biosynthesis of δ-tocotrienol and δ-tocopherol. The first two genes, which are needed for the formation of geranylgeranylpyrophosphate and homogentisate, respectively, in *E. coli*, are not specific for the biosynthesis of vitamin E. These genes are also found in, for example, the carotenoid biosynthesis pathway (in the case of *crtE*) and the tyrosine degradation pathway (in the case of *hpd*). In order to create the artificial vitamin E biosynthesis gene cluster we have used the known biosynthesis genes *crtE* (*P. ananatis*) and *hpd* (*P. putida*), because the expression and functionality of these genes had already been shown in *E. coli* in vivo experiments.[10,27,29] Furthermore, the use of these genes demonstrates the general enzyme functionality that is needed for the vitamin E biosynthesis pathway. The more vitamin E-specific biosynthesis genes *ggh*, *hpt*, and *cyc* were first amplified and cloned from *Synechocystis*. As *cyc* from *Synechocystis* was functionally not active in the in vivo approach, the tocopherol-cyclase gene from *Arabidopsis* was used.

We found that the entire engineered pathway is functional in *E. coli*, with the sole exception of the geranylgeranylpyrophosphate reductase from *Synechocystis* PCC6803 (ChlP), which had been shown earlier to be essential for the formation of tocopherol in *Synechocystis*.[13a]

The tocochromanol synthesis requires the combination of two anabolic pathways: the shikimate and the isoprenoid pathway. Therefore, a recombinant host strain should be able to deliver compounds such as FPP and HPP. The expression of the *hpd* gene from *P. putida* in *E. coli* allowed the conversion of endogenous *p*-hydroxyphenylpyruvate into HGA with molecu-

lar oxygen. *p*-Hydroxyphenylpyruvate was generated from the shikimate pathway when cells were cultivated in minimal medium, even without addition of tyrosine. When L-tyrosine was added to the minimal medium, or when tyrosine-rich LB medium was used, cultures of *E. coli* DH5α/pCAS2JF showed increased formation of HGA (up to 2 mM) in the culture supernatant. This was already an indication that *E. coli* expressing *hpd* produces sufficient homogentisate as precursor for the in vivo tocochromanol synthesis. Because HGA in the medium is easily oxidized and polymerized,[17] the actual productivity might be even higher. Recombinant CrtE in *E. coli* cells led to the formation of the second vitamin E precursor—GGPP—from FPP (188 nM GGPP in the culture), it being found almost exclusively inside the cells. The limited supply of isoprenylpyrophosphate compounds by *E. coli* has been described in other heterologous syntheses of, for example, lycopene or β-carotenoids.[19] The availability of FPP could be increased by the combined enhanced expression of the key enzymes DXS, IDI, and IspA,[20] or by the utilization of a recombinant mevalonate pathway.[21] We are currently examining whether this strategy is also successful in the case of tocochromanol biosynthesis in *E. coli*.

The assembly of the biosynthesis genes into an expression plasmid pJF119ΔN mimics gene cassettes of other natural products in which the anabolic genes are clustered or organized in operons in prokaryotic microorganisms to ensure a simultaneous expression of all cloned genes.

When the genes *crtE* and *hpd* were expressed in *E. coli* together with the genes *chlP* (*ggh*) and *hpt* from *Synechocystis,* we expected to detect MPBQ. Instead, we identified MGGBQ in the cell extracts. This suggests that GGH is not active under the given conditions, although we could detect the mRNA transcript and the recombinant protein. Alternatively, the prenyl-transfer reaction catalyzed by HPT on GGPP might be faster than the GGH/ChlP reaction on the common substrate GGPP. Indeed, HPT from *Synechocystis* has been described to accept PPP and—with lower efficiency—GGPP as prenyl donor substrate.[14c] *Synechocystis* mutants with a deletion of *chlP* had shown an increased tocotrienol content.[13a] Furthermore, in recombinant *Synechocystis* cells expressing the *hpd* gene from *Arabidopsis*, the tocotrienol amount could be increased from less than 2% to up to 20% of the total tocochromanol production. This points to a severe limitation of tocotrienol production by the homogentisate supply in *Synechocystis*.[22] Similarly, in recombinant tobacco plants, an increased supply of *p*-hydroxyphenylpyruvate and homogentisate had led to the formation of tocotrienols.[23] In our case, HGA was present abundantly in recombinant *E. coli* cells. This could be an explanation for the predominant synthesis of MGGBQ by the *Synechocystis* HPT enzyme in our cultivations.

ChlP has been shown to be a multifunctional geranylgeranyl reductase that reduces both GGPP and geranylgeranyl-chlorophyll a in *Synechocystis*.[13a] ChlP shows 67% amino acid sequence identity to the mature GGH from *Arabidopsis thaliana*.[13b] Our attempts to identify the product, PPP, in cell pellets or in supernatants from a strain expressing only *crtE* and *chlP* failed.

Through the expression of the tocopherol/tocotrienol cyclase from *Arabidopsis* (*vte1*)[18a] in conjunction with the genes for MGGBQ production, we were able to detect the in vivo production of a tocochromanol (δ-tocotrienol) in *E. coli* cells for the first time. We observed that both MGGBQ and also δ-tocotrienol were found almost exclusively in the cell pellet. The lipophilic tails of both molecules are presumably located in the cell membranes. MGGBQ concentrations were about 15 times as high as those of δ-tocotrienol (in cultures of DH5α/pCAS47), pointing to a limitation of the tocopherol cyclase step in this in vivo approach. In vitro experiments, in contrast, showed almost complete conversion of MGGBQ by VTE1 (Figure 4); this adds MGGBQ to the substrate list of recombinant VTE1, which had earlier been shown to cyclize the dimethylated phytyl- and geranylgeranylhydroquinones.[18a] The stronger decrease in the MGGBQ concentration in comparison with the increase in that of δ-tocotrienol in prolonged cultivation of *E. coli* DH5a/pCAS47 is presumably caused by its tendency to be oxidized by molecular oxygen and the subsequent polymerization of MGGBQ, as observed for its structural analogue, HGA.[17] One reason for the low yield of δ-tocotrienol in vivo could be the partial localization of the produced MGGBQ in the outer membrane of the cell, where it would be inaccessible to the cytosolic VTE1 enzyme. Furthermore, the periplasm and the outer membrane present oxidizing environments, which might support the oxidation and polymerization of MGGBQ.

In this report we show the production of δ-tocotrienol in nonphotosynthetic microorganisms—in recombinant *E. coli* cells, for example—for the first time. δ-Tocochromanol was not focused on simply because the pathway requires only four or five additional enzymatic steps in *E. coli*. The formation of δ-tocotrienol takes place without a methyltransferase reaction, which is required in the biosynthesis of α-, β-, and γ-tocochromanols. The δ-tocochromanols are by far the rarest natural vitamin E compounds and show biological activity that differs in some cases from that of the abundant α-tocopherol.[6]

Furthermore, we have identified the product of each enzymatic step from the precursors *p*-HPP and FPP towards δ-tocotrienol by in vivo experiments and have thereby reconfirmed the biosynthetic tocochromanol pathway that has been elucidated in *Synechocystis* and *Arabidopsis*.[9b]

These results offer the opportunity for further expansion of the pathway towards α-tocochromanol compounds through the additional expression of the MGGBQ- and the tocotrienol-methyltransferases. For high-level production of vitamin E in *E. coli*, more precise adjustment of the gene expression and the enzyme activities are needed in order to avoid the accumulation of pathway intermediates, as well as an increased supply of precursor molecules, in particular FPP. In addition, the described results set the stage for in vitro evolution processes to optimize enzyme activities for increased production of vitamin E in engineered plants.

Experimental Section

Chemicals: Homogentisic acid was purchased from Fluka. Geranylgeranyl pyrophosphate and δ-tocopherol was purchased from

Sigma–Aldrich. δ-Tocotrienol was purchased from Davos Life Science Pte Ltd. (Singapore). All the solvents used in this study were HPLC grade and were purchased from Carl Roth, GmbH & Co. KG (Karlsruhe, Germany) or from VWR International (Leuven, Germany)

Media and culture conditions: Luria–Bertani (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) was used in cultivation experiments for HGA production. In some experiments for HGA production, minimal medium (MM) was used; this contained KH_2PO_4 (3 g L^{-1}), K_2HPO_4 (12 g L^{-1}), $(NH_4)_2SO_4$ (5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.3 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.015 g L^{-1}), NaCl (0.1 g L^{-1}), glucose monohydrate (5 g L^{-1}), $FeSO_4 \cdot 7H_2O$/sodium citrate [15 mL L^{-1}; from a solution of $FeSO_4 \times 7H_2O$ (7.5 g L^{-1}) and sodium citrate (100 g L^{-1})], thiamine, trace elements[24] (33 mL L^{-1})[1], optionally supplemented with L-tyrosine (0.25 g L^{-1}). LB medium supplemented with glycerol (2%, *v*/*v*) and ampicillin (100 μg mL^{-1}) was used for cultivation of MGGBQ and δ-tocotrienol production. For growth curve experiments for MGGBQ and δ-tocotrienol production, medium (600 mL) in a 3 L Erlenmeyer flask without baffles was inoculated with overnight culture. The overnight culture (1%, *v*/*v*) was used as inoculum. Each strain was cultivated in two parallel flasks. Cells were grown at 30 °C with shaking at 100 rpm. Cultures were induced with isopropyl β-D-thiogalactopyranoside (IPTG, final concentration at 1 mM) at an OD_{600} of 0.8.

Cloning of the biosynthesis genes in *E. coli*: In order to create an artificial gene cluster (tocochromanol gene cassette) for expression in *E. coli* DH5α (Invitrogen, Karlsruhe, Germany), the following cloning strategy was used. Genes were cloned after appropriate PCR amplification into the expression vector pJF119ΔN. pJF119ΔN was constructed from pJF119EH,[25] by elimination of the NdeI site (by NdeI restriction, treatment with Klenow fragment, and religation) and concomitant (subsequent) insertion of an artificial Shine–Dalgarno sequence and a downstream NdeI restriction site. Modified PCR primers were used to create a BglII and an NdeI restriction site upstream of the start codon of the gene, plus an artificial RBS and a BamHI or a BglII site downstream of the stop codon. Pwol DNA polymerase (Genaxxon, Biberach, Germany) was used for amplification. The resulting PCR products were digested by the restriction enzymes NdeI and BamHI and ligated with NdeI plus BamHI hydrolyzed expression vectors (pJF119ΔN or pAW229),[25,26] or were hydrolyzed with BglII plus BamHI and ligated into the BamHI hydrolyzed expression vector (pJF119ΔN or pAW229). The *hpd* gene was amplified from chromosomal DNA of *Pseudomonas putida* KT2440 (Genbank accession no. AAN69035) with use of the following primers (upstream BglII and NdeI and downstream BamHI or BglII engineered restriction sites are underlined): CGC TGC AGA TGA GAG ATC TCA TAT GGC TGA TAT C and ATG GGA TCC TCC TTC TCA GTC TGT GCT CAG CAC GC. *crtE* was amplified from plasmid pCAR16[27] by using the primers CCG TTT ATA AGG AGA TCT CAT ATG ACG GTC TGC GC and ATG GGA TCC TCC TTC TTA ACT GAC GGC AGC GAG TTT. *ggh* (*chlP*, alias *slL1091*) was amplified from chromosomal DNA of *Synechocystis* PCC6803 (Genbank accession no. BA000022) by using the primers GAA ATT TAG GAG ATC TCA TAT GGT ATT ACG GGT AGC AGT CGT TGG and ATG GGA TCC TCC TTC TTA AGG GGC TAA AGC GTT ACC CCG GAG C. *hpt* (alias *slr1736*)[13c,14c] was amplified from chromosomal DNA of *Synechocystis* PCC6803 (Genbank accession no. BA000022) by using the primers CTT TAA GAA GGA AGA TCT CAT ATG GCA ACT ATC CAA GC and ACC TAA TTT TTC TAA TAC TAT TTT TTA GGA AGG AGG ATC CCA T. *cyc* was amplified from chromosomal DNA of *Synechocystis* PCC6803 (Genbank accession no. BA000022) by using the primers CAT AAA TTC TCA GAT CTC ATA TGA AAT TTC CGC CC and GGC GGA TCC TCC TTC TCA GAA TGG CAC TGT TTT TT. *vte1* from *Arabidopsis thaliana* was amplified from plasmid pQE31-vte1[19a] by using the primers GGT GGA TAG ATC TCA TAT GAG AGG ATC TCA CCA TCA CC and CCT AGA TCT TCC TTC TTA CAG ACC CGG TGG CTT GAA G. The PCR product of *vte1* was digested with BglII and ligated into BamHI-digested pCAS29. Restriction digestions and DNA sequencing were performed for all constructs to verify the orientations and sequences of the cloned biosynthesis genes.

Extraction and analysis of MGGBQ and tocotrienol from *E. coli* cultures: Samples (50 mL) were withdrawn from cultures, and cells were harvested by centrifugation at 2200 *g* for 30 min at 4 °C. The cell pellet was suspended in analytical grade acetone (35 mL), and the mixture was shaken vigorously at room temperature overnight. This suspension was centrifuged at 2200 *g* for 30 min at 4 °C. Acetone in the clear supernatant containing the lipophilic compounds was evaporated in a vacuum rotary evaporator, and the residue was dissolved in acetonitrile (1 mL). This dissolved residue was injected into HPLC for quantification of MGGBQ and δ-tocotrienol. HPLC analyses were performed on a Dionex HPLC Instrument, Germany, fitted with Chromeleon Software, Gina autosampler, P580 pumps, and a detector with a UV lamp. Products were analyzed by loading the samples onto a RP18 Lichrospher100 (5 μm particle size) analytical column (250×4.6 mm, Merck, Darmstadt, Germany) attached to a guard column containing a matrix of the same material as the column. A solvent flow rate of 0.8 mL min^{-1} was used. The solvents used were Solvent A, consisting of doubly distilled water with trifluoroacetic acid (TFA, 0.1%, *v*/*v*), and Solvent B, consisting of acetonitrile with TFA (0.1%, *v*/*v*). A total of 200 μL of cleared extract in hexane was analyzed under the following gradient conditions: equilibration conditions at 80% A/20% B; 0 to 10 min linear gradient from 80% A/20% B to 10% A/90% B; 10 to 30 min linear gradient from 10% A/90% B to 0% A/100% B; 30 to 50 min isocratic conditions 0% A/100% B; 50 min to 51 min linear gradient from 0% A/100% B to 80% A/20% B; isocratic conditions to equilibrate the column at 80% A/20% B.

Shaking flask cultivation of MGGBQ and δ-tocotrienol: Nine Erlenmeyer flasks of 3 L volume, each containing LB medium (400 mL) supplemented with glycerol (2%, *v*/*v*) and ampicillin (100 μg mL^{-1}), were inoculated with overnight culture at 30 °C. Cultures were incubated at 30 °C, 100 rpm. Cultures were harvested 50 h after induction. Cells were extracted for MGGBQ and δ-tocotrienol as described above.

Purification of MGGBQ by preparative HPLC: The cell extracts in acetonitrile containing MGGBQ and δ-tocotrienol were loaded in excess onto a RP18 Lichrosphere 100 (10 μm particle size) preparative column (250×10 mm, LICHROCART, Merck, Darmstadt, Germany) attached to a guard column. A solvent flow rate of 1.2 mL min^{-1} was used with isocratic conditions at 15% A/85% B as mobile phase. Solvent A consisted of doubly distilled water, and solvent B consisted of methanol. MGGBQ and δ-tocotrienol peaks were collected separately, and fractions collected after many loadings were pooled together. The pooled fractions were dried with the aid of a vacuum rotary evaporator, and the residue was dissolved and washed with methanol (20 mL) and again dried with a vacuum rotary evaporator. Finally, the residue was concentrated by dissolving it in methanol (1 mL). Half of the purified MGGBQ sample was used for the preparation of MGGBQ-cyclodextrin complex substrate used during the in vitro enzyme activity test of VTE1.[28]

Liquid chromatography-mass spectroscopy (LC-MS): LC-MS was performed on an API-ES (Agilent 1100 Series, USA). Mass fragmen-

tation spectra of the extracted samples and δ-tocotrienol standard were monitored over a mass range (*m/z*) of 200 to 800.

Analysis of the precursor molecules HGA, FPP, and GGPP: HGA produced during cultivation was released into the medium. Culture (1 mL) was withdrawn as a sample at different time intervals during cultivation. The sample was centrifuged at 14000*g* for 10 min. The clear supernatant was injected onto HPLC for HGA analysis. The same solvent system as used for MGGBQ and δ-tocotrienol analysis was used. The following HPLC flow gradient was used: 0 to 5 min equilibration conditions at 100% A/0% B; 5 to 30 min linear gradient from 100% A/0% B to 70% A/30% B; 30 to 31 min linear gradient from 70% A/30% B to 0% A/100% B; 31 to 38 min isocratic conditions 0% A/100% B; 38 min to 39 min linear gradient from 0% A/100% B to 100% A/0% B; isocratic conditions to equilibrate the column at 100% A/0% B.

Isoprenoid compounds FPP and GGPP, being highly lipophilic, are not released into the medium during cultivation. Extraction, dephosphorylation, and quantification of farnesol and geranylgeraniol by a sensitive non-radioactive analytical method was carried out as described elsewhere.[29]

Formulation of MGGBQ/methyl-ß-cyclodextrin complex:[28] Purified MGGBQ in methanol (50 μL) after preparative HPLC (described above) was dried under nitrogen gas. The residue was mixed with cyclodextrin (2.25 mM, 1 mL), which was prepared in potassium phosphate buffer (50 mM, pH 7.0). This mixture was incubated at 40 °C for 15 min on a shaker. Then 0.5 mL of ascorbic acid (500 mM) and potassium phosphate buffer (50 mM, pH 7.0) was added. This mixture was incubated at 30 °C for 15 min with constant shaking. The substrate-cyclodextrin mixture was ready, and aliquots of 100 μL each were stored at −20 °C.

Enzyme assay of γ-TC with MGGBQ: Purified His-Tag TC proteins were used for in vitro TC activity assay. *E. coli* M15 cells harboring repressor plasmid pREP4 (Qiagen, Hilden, Germany) were transformed with plasmid pVTE1 (pQE31 vector carrying *vte1* from *Arabidopsis thaliana*). As control, *E. coli* M15/pREP4 cells were transformed with plasmid pQE31 (control vector). The two resulting recombinant strains were each cultivated in LB medium in the presence of ampicillin (100 mg L^{-1}) and kanamycin (25 mg L^{-1}). Cultures were induced at an OD_{600} of 0.8 with IPTG (final concentration 1 mM) and incubated at room temperature for another 12 h. Cultures were then put on ice for 1 h and harvested at 2200*g* for 15 min. Cells were suspended in lysis buffer, lysozyme (30 μg mL^{-1}) was added, and the system was incubated for 15 min on ice. Cells were sonicated with an ultrasonic disintegrator for six periods of 15 s, with intermediate cooling on ice/water for 15 s, and at 50% amplitude. Further purification steps were carried out as described by Qiagen (Hilden, Germany) in Qiagen Expressionist, 5th edition in protocol 9 for preparation of clear *E. coli* lysate under native conditions. His-tag purification was carried out as described in protocol 16. The flow-through fraction, washed fraction, and eluted fraction were loaded on SDS-PAGE to check the protein purity.

Purified TC protein solutions (100 μL, containing 10 μg protein) were assayed for TC activity in a 1000 μL reaction mixture consisting of potassium phosphate, (pH 7.3, 200 mM), dithiothreitol (4 mM), ascorbic acid (75 mM), and formulated MGGBQ (approximately 18 μM;[28] MGGBQ concentration was based on the calculation from the reaction stoichiometry). Reaction mixtures were incubated at 30 °C. A sample (200 μL each) was taken after 0, 0.5, 2.0, 11, and 35 h. The reaction was stopped by the addition of ethanol (200 μL). Hexane (200 μL) was added to extract the lipophilic compounds from the reaction mixture, which was separated into two phases; the upper organic phase was then injected onto HPLC for analysis of unreacted MGGBQ and produced δ-tocotrienol. The same solvent system as used for extraction and analysis of MGGBQ and δ-tocotrienol from *E. coli* cultures was used. Equilibration conditions at 30% A/70% B; 0 to 10 min linear gradient from 30% A/70% B to 10% A/90% B; 10 to 40 min linear gradient from 10% A/90% B to 0% A/100% B; 40 to 70 min isocratic conditions 0% A/100% B; 70 min to 71 min linear gradient from 0% A/100% B to 30% A/70% B; isocratic conditions to equilibrate the column at 30% A/70% B from 71 min to 78 min. The retention times for HPLC peaks 1 and 2 differ during the in vitro analysis (Figure 4A) and in vivo analysis (Figure 5) because of the different HPLC conditions and different solvents used for dissolving the samples. The in vitro samples analyzed by HPLC were dissolved in hexane, and the in vivo samples were dissolved in acetonitrile.

Estimation of MGGBQ concentration: MGGBQ produced in vivo could not be quantified, because no MGGBQ analytical standard could be purchased commercially. To calculate the approximate concentration of MGGBQ, a simple calculation based on the reaction stoichiometry (Scheme 1) was performed, with 1 mole of MGGBQ yielding 1 mole of δ-tocotrienol. This calculation was done for the in vitro experiment carried out with purified MGGBQ and purified TC proteins. The calculation was based on the assumption that the product yield was 100% (that is, all MGGBQ consumed during the in vitro activity test was converted into δ-tocotrienol).

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